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Displacement chromatography of isomers and therapeutic compounds

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Abstract

Displacement chromatography was successfully used to separate a binary isomer mixture, epirubicin and doxorubicin, on Kromasil KR100-10 C_{18} 250×4.6 mm I.D. (10 μm) column. Displacement parameters such as the types and the concentrations of displacer, the composition and the flow rate of the mobile phase were critically examined in this study. The displacer employed was 30 mg/ml benzethonium chloride. Loading of feed at lower initial organic level of mobile phase coupled with displacement at higher organic level was found to give efficient separation. A 30-mg amount of binary isomer mixture was separated on an analytical column. The purification of epirubicin from the closely related impurities present in raw product solution by displacement chromatography was also investigated. The purity of epirubicin required was greater than 99% with a recovery of 60%. The results have indicated that this process made good use of the high feed load, low solvent costs, and high resolution characteristics of displacement chromatography and offered the chromatographic engineer a powerful tool for the preparative purification of therapeutic compounds. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Displacement chromatography; Preparative chromatography; Epirubicin; Doxorubicin

1. Introduction

Preparative and process scale separation of therapeutic compounds has been of considerable interest in recent years [1]. In current practice, preparative chromatography is almost exclusively carried out in the overload elution mode. It has been suggested, however, that displacement chromatography, an intrinsically nonlinear mode, has several advantages over the elution mode in process scale separations [2–16]. It may offer the use of larger sample sizes, lower solvent requirements, the recovery of products at relatively high concentration, easy control of the speed and efficiency of process with the displacer serving as an additional separating agent. The displacement mode of chromatography, introduced by Tiselius in 1943, was revived by Horvath [3], and has since found many applications to the separation of biomolecules. Recent reviews have discussed the fundamentals and practice of displacement chromatography [17–19].

Displacement chromatography has also been recognized as a method that is especially suited to the separation of closely related substances that differ only minimally in the stationary phase affinity. One area where this resolving power may benefit the

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biotechnical and pharmaceutical industry most directly, is that of the separation of optical and structural isomers. Vigh and co-workers used cyclodextrin–silica columns for the separation of positional and geometrical isomers [20–22]. Horvath et al. examined the potential of displacement chromatography for the separation of isomers by simulating the separation of interconverting species under various conditions. The results were expected to aid the optimization and scale-up of the displacement chromatography [23].

Epirubicin (4′-epidoxorubicin), an anthracycline antitumor antibiotics, is an isomer of doxorubicin modified in the sugar moiety. They have been found to be useful in the treatment of cancer [24]. In this paper, reversed-phase displacement chromatography was used to separate a binary isomer mixture, epirubicin and doxorubicin on a Kromasil KR100-10 C_{18} 250×4.6 mm I.D. (10 μm) column. Displacement parameters, such as the choice of displacer, displacer concentration, the composition and the flow rate of the mobile phase were critically examined.

To date, academic researchers have been the main advocates of displacement chromatography, therefore most reported examples involve model product systems. For displacement chromatography to have utility for biopharmaceutical downstream processing, it must be able to separate some rather difficult "real world" mixtures [5,11]. It was reported that rHuBDNF was purified from impurities that have extremely similar chromatographic behavior in a single step by cation-exchange displacement chromatography [25]. More recently, displacement chromatography has been suggested as an economically sound way for the large-scale purification of oligonucleotides [26]. Therefore, the purification of epirubicin from the impurities present in a raw product solution by displacement chromatography was also investigated. The results have demonstrated that making use of displacement chromatography for the purification of therapeutic compounds is attractive.

2. Experimental

2.1. Materials

Kromasil KR100-10 C_{18} 250×4.6 mm I.D. (10 μm) column was obtained from Eka Chemicals (Bohus, Sweden). Benzethonium chloride was obtained from Fluka (Buchs, Switzerland). The other chemicals were of HPLC-grade or analytical pure grade. The water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Epirubicin raw product solution, epirubicin and doxorubicin standards were kindly provided by Hisun Pharmaceutical (Zhejiang, China).

2.2. Apparatus

All preparative separations and subsequent analysis of fractions were performed on TSP HPLC system (TSP, San Jose, CA, USA), which consisted of a P4000 pump, an AS3000 autosampler, a Spectra FOCUS diode array detector, and a data work station. Chromatographic system control, analysis, data acquisition and chromatographic analysis were performed with TSP Spectra System Software PC 1000 Chromatography Manager Software (3.0 version).

2.3. Procedures

2.3.1. Displacement of isomers mixture

Both the displacement separations of the samples by different displacers and the analysis of the fractions collected during displacement process were all carried out on the same column. The column was first equilibrated with a certain proportion of methanol–water (adjusted pH 2.4 with formic acid) as the mobile phase. The feed containing epirubicin and doxorubicin (1:1, w/w) in the mobile phase was then loaded into the sample loop (The mobile phase composition of feed was identical to the initial state of column in all preparative runs). After feeding, the displacer solution was pumped continuously into the column at the flow rate of 0.2 ml/min. The fractions were collected at the outlet of the UV detector and the fractions were started by turning the injection valve. After the breakthrough point of the displacer, 100% methanol was pumped through the system at the flow rate of 1.5 ml/min for 20 min to remove the displacer completely. The column was then reequilibrated with the mobile phase for the next run or analysis of the fractions collected.

2.3.2. Purification of epirubicin from raw product solution

A 2-ml volume of raw solution of epirubicin was purified by displacement chromatography using Kromasil KR100-10 C_{18} 250×4.6 mm I.D. (10 μm) analytical column. The displacer was 30 mg/ml benzethonium chloride dissolved in methanol–water (30:70, v/v, adjusted pH 2.4 with formic acid) mobile phase. The column was first equilibrated with methanol–water (20:80, v/v, adjusted pH 2.4 with formic acid). Then, the 2-ml feed loop of the sample injector was filled up with the raw solution to be separated. After the introduction of the feed, all other procedures were the same as described above.

2.3.3. HPLC analysis

The fractions collected from displacement process were analyzed using the same column with methanol–water (60:40, v/v, adjusted pH 2.4 with formic acid) as a mobile phase at the flow rate of 1.0 ml/min and room temperature, and were monitored at UV 254 nm. The data acquired from workstation for the all fractions were used to reconstruct the displacement chromatogram.

Benzethonium chloride was also analyzed using methanol–water (80:20, v/v, adjusted pH 2.4 with formic acid) as a mobile phase and detected at UV 254 nm.

3. Results and discussion

3.1. Choice of the displacer

The selection of a suitable displacer may be the most important part of establishing the conditions for displacement chromatography [27,28]. The displacer has to bind to the stationary phase stronger than any of the components of the mixture to be separated and the adsorption isotherm should be Langmuirian in the range of concentration to be concerned. In addition, it has to be sufficiently soluble in the carrier. The displacer should not interact with the feed components. Despite the required strong binding of the displacer to the adsorbent in contact with the carrier, it is also important that the displacer be prone to some scheme for ready removal in order to regenerate the column. Other requirements discussed in detail in reviews [2–4]. In our study, ethylene glycol, dimethyl sulfoxide, *n*-octyl alcohol, cetyltrimethylammonium bromide, ammonium citrate, citric acid, glycerin, iso-pentanol, benzyl tributyl ammonium chloride, and benzethonium chloride were tried. Finally, benzethonium chloride was found to be a good candidate: it is strongly retained, highly soluble, easily detected by UV, and could displace the spiked sample of epirubicin and doxorubicin and a real raw solution of epirubicin successfully.

3.2. Choice of displacer concentration

The concentration of the displacer also plays an important role for the efficiency of the separation process. There is an optimum concentration of the displacer for a given set of chromatographic conditions, therefore, the three concentrations of the displacer i.e. 12, 30, and 50 mg/ml were tested to run displacement separation of the epirubicin and doxorubicin mixture. With 12 mg/ml of the concentration of benzethonium chloride, the separation was unsuccessful even at a relatively high level of methanol (50%, v/v) in the mobile phase. Then, 30 mg/ml and 50 mg/ml of benzethonium chloride solution were selected respectively, and the results indicated that both of them could obtain a displacement train of isomer mixture successfully, as shown in Fig. 1. From the figures, it was found that at low displacer concentration (30 mg/ml), the breakthrough volumes were relatively large and the bands were moved at low speed and low concentrations. In contrast, at high displacer concentrations (50 mg/ml), the bands were moved at high speed and concentrations so that the breakthrough volume was small. The velocity of the displacer front passed through the column was increased with the higher displacer concentration, which is in accordance with the displacement theory [3]. Meanwhile, with increasing the displacer concentration, the concentration of the products in the effluent was also raised, and the bands of individual components then became narrow. However, when the concentration of the displacer reached 50 mg/ml, no pure epirubicin was acquired due to the overlap of the boundaries between the displaced zones. In terms of the maximum recovery of the pure product, 30 mg/ml was

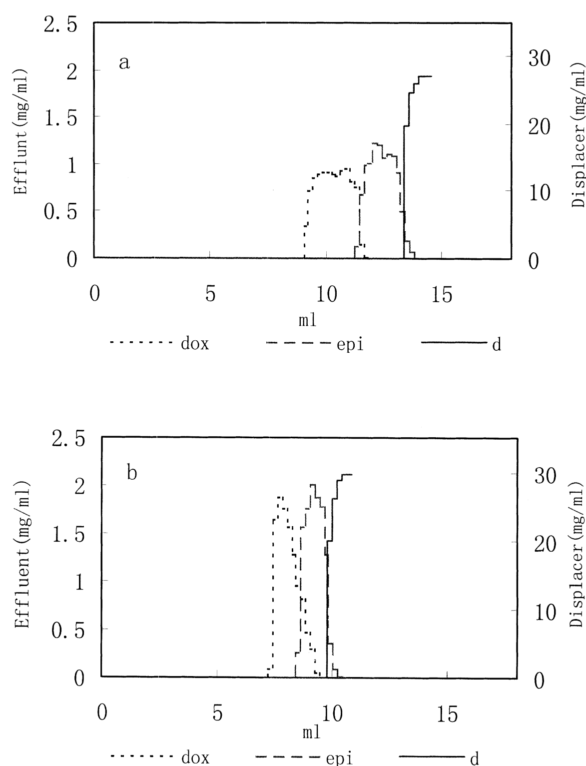


Fig. 1. Effect of the displacer concentration. Mobile phase: methanol–water (40:60, v/v, pH=2.4); Feed load: 4 mg of isomer mixture; Flow-rate: 0.2 ml/min. (a) Using 30 mg/ml of benzethonium chloride dissolved in the same mobile phase as displacer. (b) Using 50 mg/ml of benzethonium chloride dissolved in the same mobile phase as displacer.

selected as optimum concentration of the displacer in our study.

3.3. Choice of the composition of the mobile phase

The mobile phase that is conveniently called the carrier in displacement chromatography should be selected so that the feed components are sufficiently strongly retained on the stationary phase. High solubility of the feed components in the carrier is also required. Both the feed and the displacer are dissolved in the carrier, so minor differences in organic level of the mobile phase both in the initial feed state and in the displacer could cause significant differences in retention. The retention of many molecules distinctly decreases with increasing organic level in the mobile phase in reversed-phase mode.

Separation using 30 mg/ml benzethonium chloride as displacer dissolved in methanol–water (20:80, v/v, pH=2.4) for 8.1 mg feed where the initial state of column was also methanol–water (20:80, v/v, pH=2.4) is shown in Fig. 2a (hereafter 20 initial/20 displacement). By comparing this to the 8.1-mg run when both the displacer and initial state of the column were methanol–water (40:60, v/v, pH=2.4) (40 initial/40 displacement) (Fig. 2b), it can be observed that using the mobile phase with high organic levels could obviously help in reducing the separation time. However, at the high initial organic level, the feed components moved too fast which resulted in less efficient separation because of decreased selectivity. On the other hand, when low organic levels were used, the molecules bind strongly to the stationary phase, thereby increasing the load capacity, resulting in complete separation, and a highly concentrated product, but substantially increasing the separation time and then resulting in lower productivity.

Hence, the highest productivity, reflecting the balance between loading and selectivity on the one hand and the separation time on the other, will be obtained by a judicious choice of organic levels in the feed state and in the displacer. In practice, the composition of the mobile phase can differ from those of the solvents used for the feed and the displacer [2]. So, separation run when the displacer solution was dissolved at methanol–water (30:70, v/v, pH=2.4) keeping both the initial state of column and the feed state at methanol–water (20:80, v/v, pH=2.4) (20 initial/30 displacement) was also studied as shown in Fig. 2c. Comparison of Fig. 2c (20 initial/30 displacement) and Fig. 2a (20 initial/20 displacement) demonstrated that no apparent difference on separation efficiency between them, but the former led to a shorter separation time.

It can be concluded from this series of the experiments that the best results were achieved when the feed was loaded at low organic level, thereby expediting the discrimination of stationary phases for the feed component, and displacement carried out with higher organic level in the displacer solution, producing rapid separations with higher productivity. It coincides with the results in the literature [28].

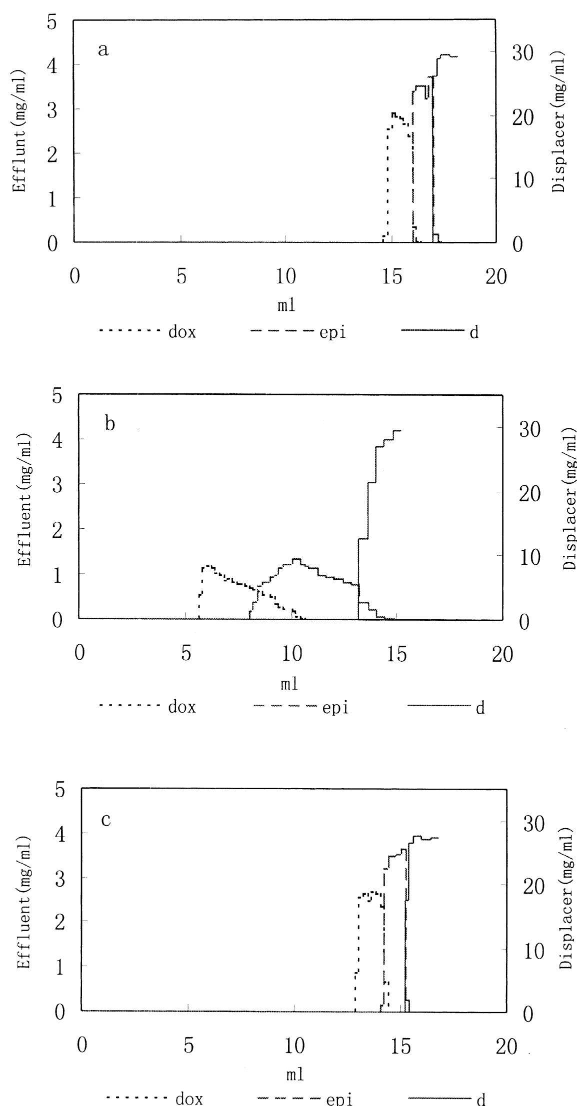


Fig. 2. Effect of the composition of mobile phase. Feed load: 8.1 mg of isomer mixture; Flow-rate: 0.2 ml/min. (a) Mobile phase: methanol–water (20:80, v/v, pH=2.4), displacer: 30 mg/ml of benzethonium chloride dissolved in the same mobile phase. (b) Mobile phase: methanol–water (40:60, v/v, pH=2.4), displacer: 30 mg/ml of benzethonium chloride dissolved in the same mobile phase. (c) Mobile phase: methanol–water (20:80, v/v, pH=2.4), displacer: 30 mg/ml of benzethonium chloride dissolved in methanol–water (30:70, v/v, pH=2.4).

Therefore, in the following preparative runs, the displacer solution was dissolved at methanol–water (30:70, v/v, pH=2.4) and both the initial state of column and the feed state were the same at methanol–water (20:80, v/v, pH=2.4).

3.4. Choice of the flow-rate

The flow rate of the mobile phase is generally lower in the displacement mode than in the elution mode, because the low flow-rate can result in improved displacement separations [2,3]. However, it is also reported that the flow-rate was not a limiting factor in a displacement run, and separation at a low flow-rate (0.1 ml/min) and high flow-rate (0.75 ml/min) were quite similar [29]. In order to test the effect of the flow-rate on the separation of epirubicin and doxorubicin isomers mixtures, displacement at the flow rate of 0.1 and 0.5 ml/min were also carried out and the experiment results were shown in Fig. 3(a and b). In comparison with the run at the flow rate of 0.2 ml/min (Fig. 1a), we can conclude that at the flow rate of 0.5 ml/min, the extent of mixing increased apparently, the band shapes were irregular. By contrast, at the lower flow rate of 0.1 and 0.2 ml/min, the much improved separations of the components were accomplished. The results indicated that 0.5 ml/min was too high to obtain a satisfactory result, although it is already lower than those typically employed in analytical HPLC. Moreover, separations at 0.1 and 0.2 ml/min were quite similar, in terms of the extent of mixing. Thus, the flow rate of 0.2 ml/min was selected.

3.5. Effect of feed loading

The column was first equilibrated with the mobile phase consisting of methanol–water (20:80, v/v, pH=2.4) followed by loading 16 and 30 mg of feed mixture dissolved in the same mobile phase. Then, the displacement step was started with the displacer dissolved in methanol–water (30:70, v/v, pH=2.4) at the end of the feed introduction. The displacement chromatograms are shown in Fig. 4(a and b). The results have demonstrated that preparative-scale separations of isomer mixtures were successfully carried out on an analytical column using 30 mg/ml of benzethonium chloride as displacer.

An overloaded elution chromatogram obtained with the same column at the sample load of 0.2 mg

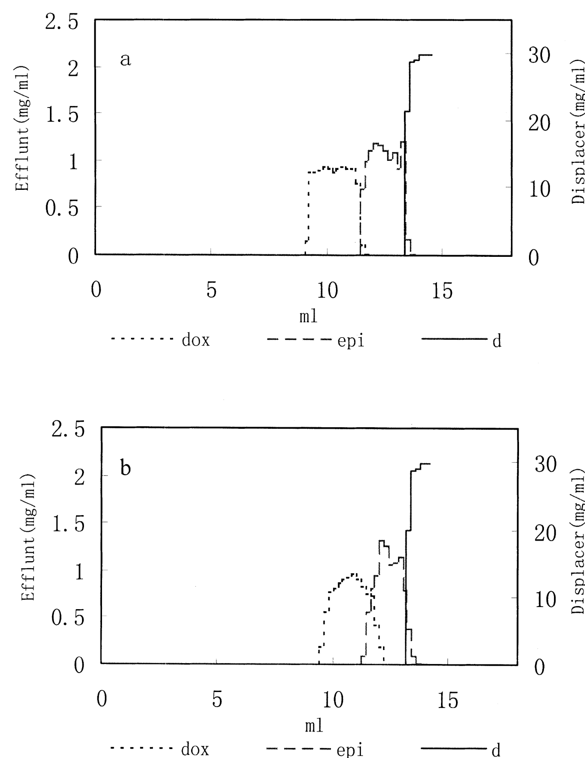


Fig. 3. Effect of the flow-rate. Mobile phase: methanol–water (40:60, v/v, pH=2.4); Displacer: 30 mg/ml of benzethonium chloride dissolved in the same mobile phase; Feed load: 4 mg of isomer mixture. (a) The flow-rate was 0.1 ml/min. (b) The flow-rate was 0.5 ml/min.

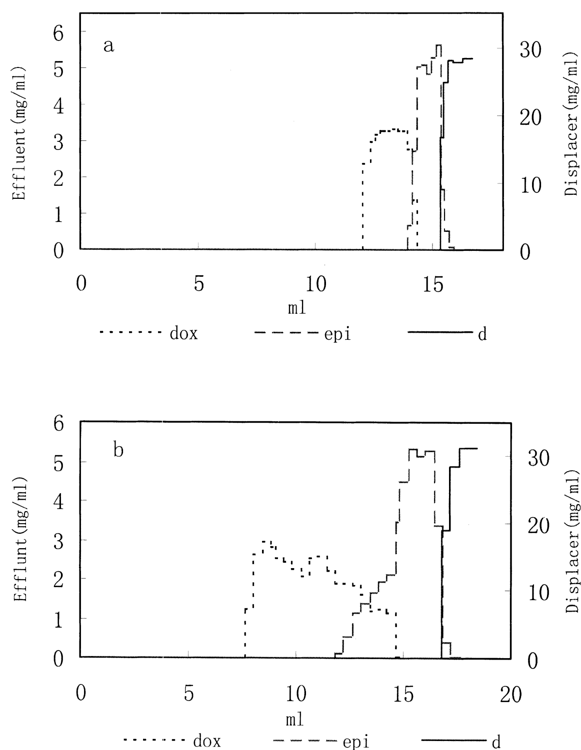


Fig. 4. Effect of feed loading. Mobile phase: methanol–water (20:80, v/v, pH=2.4); Displacer: 30 mg/ml of benzethonium chloride dissolved in methanol–water (30:70, v/v, pH=2.4); Flow-rate: 0.2 ml/min. (a) 16 mg feed loading. (b) 30 mg feed loading.

is shown in Fig. 5. A comparison of the sample load with both elution chromatography and displacement chromatography has strikingly demonstrated the differences between these two modes of chromatographic development, and the superiority of the displacement mode. We can conclude that the chromatographic system with analytical columns in the displacement development can be used for the separation of preparative amounts of relatively close mixtures. The high loading capacity in displacement chromatography is the result of the better utilization of the column and equipment than possible in linear elution chromatography where not only the solute concentration has to be low but also a large fraction of the column volume between the peaks is idle. Besides, the larger volume of the fraction collected from the column run with elution mode needs more time for past treatment of the products.

As the feed loading increased from 8.1 to 16 and 30 mg, the acquired amounts of the pure doxorubicin and epirubicin, and their percentages are summarized in Table 1. With increasing the feed loading, the percentage of acquired pure product was decreased from 74, 68 to 63% for doxorubicin, and from 72, 65 to 54% for epirubicin. It has shown that the mixed zones of epirubicin and doxorubicin increased. However, the absolute amount of pure epirubicin and doxorubicin also increased at the same time, 9.49 and 8.14 mg as maximum amounts for pure doxorubicin and epirubicin were reached respectively by loading 30 mg of the feed. Moreover, under the same chromatographic condition, a further increase of the feed load can not apparently increase the concen-

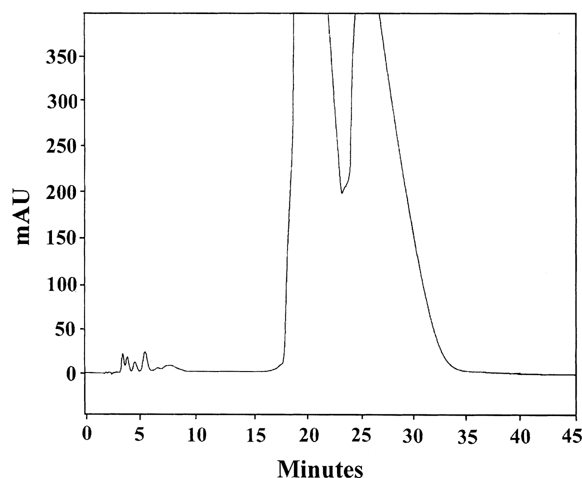


Fig. 5. Chromatogram illustrating the overloading in elution chromatography of doxorubicin and epirubicin mixture. The mobile phase was methanol–water (40:60, v/v, pH=2.4); The flow-rate was 1.0 ml/min; UV detection was 254 nm; Feed load: 0.2 mg of isomer mixture (1:1, m/m).

tration of the fractions, which is controlled by the operating line in displacement chromatography. A further increase of sample loading requires a longer column in this case. The reason is that when the displacer solution is pumped into the column, the sharp front boundary of the displacer causes the feed components to be desorbed from the surface of the stationary phase and to move down the column ahead of the displacer front. The mutual competition of the feed components for the adsorption sites brings about the separation so that they reach isotachic conditions and form a displacement train of adjacent bands of the pure components. When the length of the column is not long enough for greater sample load, no complete separation occurs at the outlet of the column, and the displacement train can not be fully developed.

3.6. Displacement separation of "real and practical" sample

To date, academic researchers have been the main advocates of displacement chromatography. Therefore, most reported examples involve model product systems. In order to have utility of displacement chromatography for biopharmaceutical downstream processing, it must be possible to separate complex "real and practical" mixtures. It is reported that one of the principal advantages of displacement chromatography is the high resolution that can be achieved. Such high resolutions are required at several stages in the downstream processing, particularly for the removal of closely related impurities of the product [30]. Accordingly, the purification of epirubicin, an anthracycline antitumor antibiotic, from a raw product solution by displacement chromatography was also investigated.

An analytical chromatogram of raw product solution on Kromasil KR100-10 C_{18} 250×4.6 mm I.D. (10 μm) column is shown in Fig. 6. It can be seen in the figure that the raw solution consisted of the main peak of epirubicin along with several closely related impurity peaks such as doxorubicin, daunorubicin and epi-daunorubicin eluted before and after the main peak. A 2-ml raw solution of 81.0% purity was injected into the system for displacement separation according to the procedure mentioned before. The fractions were collected and the concentrations of the epirubicin and displacer were analyzed. The reconstructed displacement chromatogram is shown in Fig. 7. HPLC analysis of pooled fractions and representative early, middle, and late fractions of the displace-

Table 1
Effect of the feed load

Feed load (mg)	The amount of the pure doxorubicin (mg)	Percentage (%)	The amount of the pure epirubicin (mg)	Percentage (%)
8.1	2.97	74	2.87	72
16	5.46	68	5.22	65
30	9.49	63	8.14	54

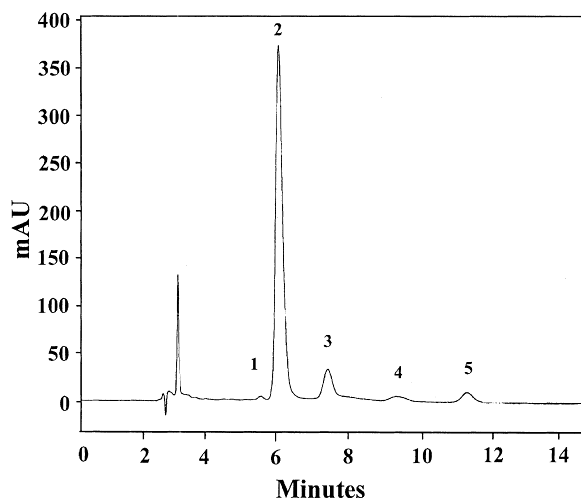


Fig. 6. Analytical chromatogram of raw product solution. Mobile phase: methanol–water (60:40, v/v, pH=2.4); The flow-rate was 1.0 ml/min; UV detection was 254 nm; Sample: 20 μl raw product solution diluted 1:10 with mobile phase. Peak identification: 1=doxorubicin, 2=epirubicin, 3=unknown impurity, 4=daunorubicin, 5=epi-daunorubicin. Other conditions as given in the text.

ment train are shown in Fig. 8. The early fractions (Fig. 8a) showed higher concentrations of the impurities that eluted before the main peak in the assay, and the later fractions (Fig. 8c) showed increasing levels of the later eluting impurities. The representative middle fractions (Fig. 8b) and the pooled fraction (Fig. 8d) illustrated the high purity that could be achieved by this process. The separation

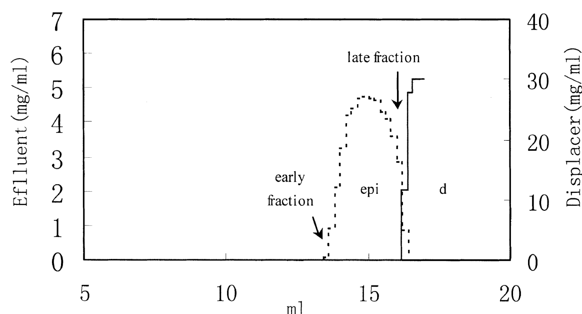
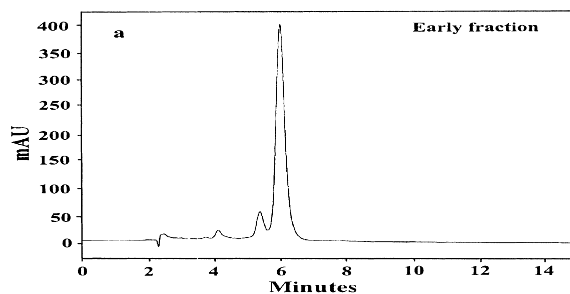
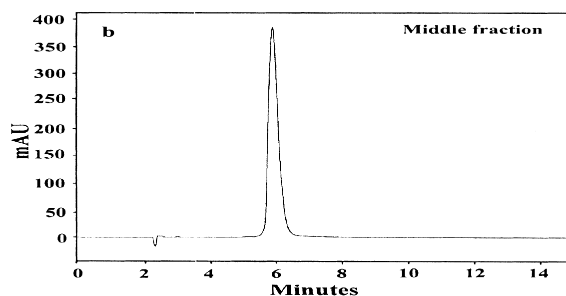
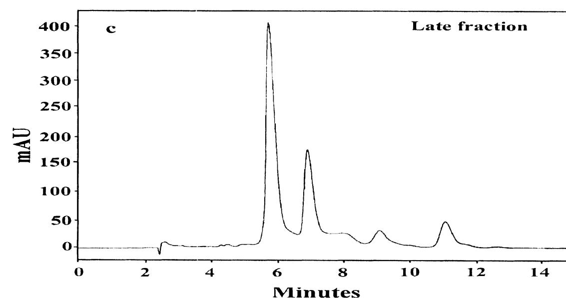


Fig. 7. Displacement chromatogram of epirubicin raw product solution. Mobile phase: methanol–water (20:80, v/v, pH=2.4); Displacer: 30 mg/ml of benzethonium chloride dissolved in methanol–water (30:70, v/v, pH=2.4); Flow-rate: 0.2 ml/min. Feed load: 2-ml epirubicin raw solution.







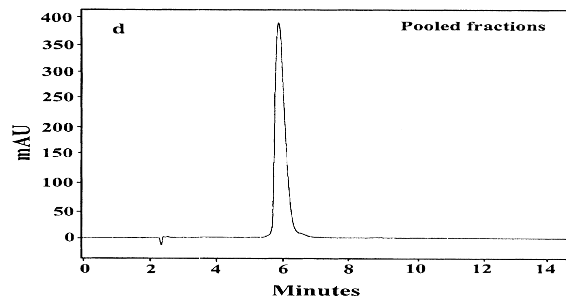


Fig. 8. Analytical chromatogram of the pooled fraction and fractions from the early, middle, and late part of the displacement train. Analytical conditions as shown in Fig. 6.

resulted in 99.13% of the epirubicin purity with a recovery of 60%.

This experiment has demonstrated that displacement chromatography could be used in the purification of a desired feed component from a relatively complex raw product mixture. Furthermore, if there is only one compound of interest, the displacement chromatography can be tailor-made to displace the desired compound. Importantly, the solvent concentration used for the process (20% methanol) is considerably lower than that required for elution mode. Thus, displacement chromatography can potentially result in lower solvent costs and may obviate the need for production facilities. All of these advantages make use of displacement chromatography for the purification of therapeutic compounds in HPLC systems particularly attractive.

4. Conclusion

Reversed-phase displacement chromatography using 30 mg/ml of benzethonium chloride as displacer was shown to be an effective means for purification of a binary isomer mixture in a preparative scale on analytical column. The high resolutions were also achieved for ‘‘real world’’ mixture, epirubicin from its closely related impurities present in the raw product. Displacement mode has shown to be of high efficiency and great save in the solvent requirements in the process of purification, and offered the possibility of simultaneous high resolutions and high loading. It has also demonstrated again that high-performance displacement chromatography, as one of the effective methods has been more and more attractive for the purification of therapeutic compounds in industrial processes.

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